Stem Cell Research (2009) 3, 28–38

available at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/scr

REGULAR ARTICLE

Analysis of the distinct functions of growth factors and tissue culture substrates necessary for the long-term self-renewal of human embryonic stem cell lines☆

Melissa A. Baxter [a], Maria V. Camarasa [b], Nicola Bates [b], Fiona Small [c], Patricia Murray [d], David Edgar [b,d], Susan J. Kimber [a,b,*]

[a] *Faculty of Life Sciences, The University of Manchester, 2nd Floor CTF Building, Oxford Road, Manchester, UK M13 3PT*
[b] *North West Embryonic Stem Cell Centre, Ground Floor CTF Building, Faculty of Life Sciences, Oxford Road, Manchester, UK M13 3PT*
[c] *North West Regional Cytogenetics Unit, St Mary's Hospital, Manchester Royal Infirmary, Oxford Road, Manchester, UK M13 9WL*
[d] *The University of Liverpool Schools of Biological and Biomedical Sciences, Liverpool, UK L69 3BX*

Received 9 January 2009; received in revised form 20 March 2009; accepted 20 March 2009

Abstract The role of individual supplements necessary for the self-renewal of human embryonic stem (hES) cells is poorly characterized, and furthermore we have found that previously reported feeder cell- and serum-free culture systems used for individual hES cell lines are unable to maintain HUES7 cells for more than one passage. We have therefore derived a feeder/serum-free culture system that can support the long-term (at least 10 passages) self-renewal of several euploid hES cell lines including MAN1, HUES7, and HUES1 with minimal spontaneous differentiation and without the need for manual propagation. This system contains fibroblast growth factor 2, activin A, neurotrophin 4, and the N2, B27 supplements together with a human fibronectin substrate. We demonstrate that these components exert distinct functions: both FGF2 and activin A were necessary to prevent differentiation of hES cells while NT4 promoted cell survival, FGF2 could not be substituted by IGFII, and the fibronectin substrate supported a rapid rate of hES culture expansion. Inhibition studies showed that β1 integrin-dependent attachment of hES cells to fibronectin was at least partially via the α5 subunit but independent of integrin αV.

☆ This work was supported by the Biotechnology and Biological Sciences Research Council [Grant BB/D014638/1] and the North West Development Agency.
* Corresponding author. 2nd Floor, Core Technology Facility, Faculty of Life Sciences, The University of Manchester, Oxford Road, Manchester, UK M13 9PT. Fax: +44 161 275 5600.
E-mail addresses: Melissa.baxter@manchester.ac.uk (M.A. Baxter), Maria.camarasa@manchester.ac.uk (M.V. Camarasa), Nicola.bates@manchester.ac.uk (N. Bates), Fiona.small@CMMC.nhs.uk (F. Small), p.a.murray@liverpool.ac.uk (P. Murray), Dhedgar@liverpool.ac.uk (D. Edgar), sue.kimber@manchester.ac.uk (S.J. Kimber).

1873-5061/$ – see front matter
doi:10.1016/j.scr.2009.03.002

Introduction

The most reliable and robust method used to maintain pluripotent human embryonic stem (hES) cells in tissue culture is by coculture on mitotically inactivated mouse feeder cells (MEFs) (Thomson et al., 1998; Amit et al., 2000). However, hES cells cultured under these conditions are exposed to xenoantigens from the feeder cells and accumulate animal cell products including the sialic acid Neu5Gc (Martin et al., 2005) and bovine apolipoprotein B-100 (Hisamatsu-Sakamoto et al., 2007). To get around the potential zoonotic pathogen and xenoantigen problems, a variety of human feeder cells including adult and fetal-derived fibroblasts (Richards et al., 2002, 2003; Inzunza et al., 2005) and autogenic fibroblasts derived from hES cells (Stojkovic et al., 2005) have been used to support the derivation and maintenance of hES cells (Richards et al., 2002). However, the properties of primary feeder cells are subject to donor to donor variation (Richards et al., 2003) and the undefined factors they provide hamper experimental analysis.

As an alternative to feeder cells, hES cells have been cultured on either Matrigel (BD, Oxford, UK) (Yao et al., 2006) or fetal bovine serum (Vallier et al., 2005). Although human serum has also been used there is evidence that it contains factors that can induce hES cell differentiation during long-term culture (Richards et al., 2003; Rajala et al., 2007). While such culture additives have been used with some success, they are still poorly defined animal derivatives; Matrigel is a mixture of animal basement membrane components and growth factors extracted from the EHS mouse sarcoma cell line (Kleinman et al., 1982), while sera contain components including growth factors, extracellular matrix molecules, and hormones, many of which are still undefined. In 2004, Amit et al. described a serum-free medium containing serum replacement that was supplemented with both FGF2 and TGFβ1. This medium was able to support hES cells on a human fibronectin substrate (Amit et al., 2004). However serum-replacement supplements are not fully defined and have also resulted in spontaneous hES cell differentiation (Xu et al., 2005).

Improved long-term hES cell maintenance has been achieved using MEF conditioned medium (Xu et al., 2001) or defined media that include growth factors produced by MEFs (Chin et al., 2007; Lu et al., 2006). In 2006 Ludwig et al. successfully derived two new hES cell lines using completely defined culture conditions ("TeSR1" culture medium on a human extracellular matrix substrate) but both lines became karyotypically abnormal after extended culture (Ludwig et al., 2006). It is likely that a hES cell line derived under more stringent feeder-free, defined conditions would be subject to increased selective pressure to undergo karyotypic change on culture adaption (Baker et al., 2007).

We describe here the validation of a fully defined, feeder and serum-free culture system that supports the extended self-renewal of pluripotent, karyotypically normal multiple hES cell lines with minimal spontaneous differentiation. The components of this system are derived from a combination of previously published techniques (Vallier et al., 2005; Liu et al., 2006) that individually are unable to support the long-term self-renewal of some of the available hES lines we are using. We demonstrate that it is necessary to supplement defined medium with FGF2, activin A, and neurotrophin 4 (NT4), and we analyze the roles of each of these factors on hES cell behavior. Furthermore, we demonstrate that human fibronectin acts specifically via α5β1 integrin to support self-renewal. Thus, this study both describes and analyses a potentially scalable culture system for the maintenance of hES cells under fully defined conditions.

Results

Growth of HUES7 hES cells under previously described conditions

HUES7 hES cells were cultured on MEF layers in medium containing KSR and passaged with trypsin approximately every 7 days according to the HUES Cell Collection Instruction Manual (HUES Cell Collection Instruction Manual). On feeders HUES7 cells formed distinct colonies that displayed nuclear OCT4 immunostaining (Fig. 1a).

In order to assess hES cell growth factors and substrate requirements for maintenance and expansion, it was necessary to use more defined culture conditions without feeders and KSR. We chose initially to compare the abilities of previously published feeder/serum-free culture conditions to support the self-renewal of HUES7 hES cells. We examined the media described by Vallier et al., for H9 cells (Vallier et al., 2005), and Lui et al., for H1 and H9 cells (Liu et al., 2006). As our aim was to examine substrate–hES cell interaction, we chose to culture hES cells on a defined human plasma fibronectin substrate as described by Lui et al., rather than Matrigel, as described by Vallier et al., which is a composite substrate that includes undefined murine components.

Compared to culture on feeder cells, after 10 days culture (2 passages, cells split 1:1), few cells remained in both feeder-free systems. These did not exhibit typical hES cell morphology. . More importantly, the cells under both feeder-free conditions had started to differentiate as determined by the absence of OCT4 immunostaining (Figs. 1b, c). Thus, while the MEF feeder cells were able to support the self-renewal of HUES7 cells, the previously described feeder cell-free conditions (Vallier et al., 2005; Liu et al., 2006) failed to maintain their self-renewal.

Derivation of modified culture conditions

In order to modify and optimise a serum-free medium to support HUES7 cell self-renewal and to allow the investigation of hES cell growth factor requirements, we supplemented the medium described by Liu et al. (2006) with activin A (10 ng/ml) as described by Vallier et al. (2005). With the addition of activin A more cells survived over a longer period in culture (3 passages over 2 weeks) but the majority were $OCT4^-$, $Nanog^-$; indicating that they had begun to differentiate (Figs. 2a–c). Given that neurotrophin 4 (NT4) has been previously shown to promote H1 and H9 hES cell survival (Pyle et al., 2006), we therefore added 4 ng/ml NT4 to the combined culture medium. With the addition of NT4 hES cell

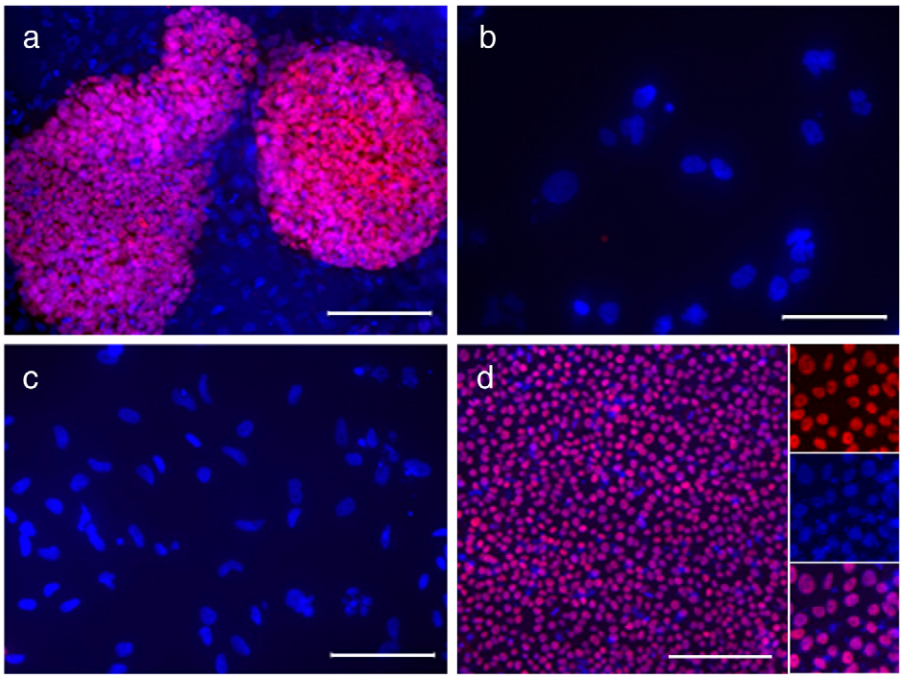

Figure 1 HUES7 cells cultured under different culture conditions: on fibronectin on MEF cells (a), under conditions described by Vallier et al., (b) and Lui et al., (c) and under our modified culture conditions; with a ×10 magnified inset of DAPI, OCT4, and merged channels (d). Immunostained for OCT4. Scale bars 100 μm.

survival increased as expected, cultures expanded rapidly (Figs. 2m, n), and importantly the majority remained OCT4$^+$ and Nanog$^+$ for 3 passages over 2 weeks (Figs. 1d, 2d–f). Therefore the combination of FGF2 and activin A, together with NT4 supplementation (Pyle et al., 2006) is necessary for the long-term maintenance of HUES7 cells under serum- and MEF-free conditions.

To establish the necessity and role of FGF2 and activin A in the presence of NT4, HUES7 were cultured for 3 passages over 2 weeks with NT4 but without either FGF2 or activin A. In the absence of activin A, HUES7 cells lost both OCT4 and Nanog expression, cultures adopted a heterogeneous morphology, and growth rates significantly decreased (Figs. 2g–i, m). Interestingly, the removal of activin A had no affect on cell survival (Fig. 2n). In the absence of FGF2 cultures also adopted a heterogeneous morphology with loss of OCT4 and Nanog expression and decreased cell survival (Figs. 2j–l). Most strikingly, the rate of culture expansion significantly decreased, indicating an important role in proliferation (Fig. 2n). Taken together, these results confirm that FGF2, activin A, and NT4 are all essential for hES cell self-renewal under these serum/feeder-free culture conditions.

Validation of modified feeder/serum-free culture conditions

HUES7 hES cell feeder cultures were dissociated using trypsin and seeded at a plating efficiency of 84 ± 3% (n = 4) onto fibronectin in the serum-free medium. This indicated that the majority of hES cells had survived from the feeder culture on the fibronectin substrate. Long-term culture demonstrated that these conditions supported the self-renewal of HUES7 cells for >10 passages cells were cultured up to 15 passages for >3 months continuous culture. The conditions were also able to support the long-term self-renewal of MAN1 and HUES1 hES cells for >10 passages (Supplemental Fig. 1). All 3 hES cell lines cultured under these conditions were successfully cryopreserved and reestablished after thawing (not shown). Morphologically all hES cell lines exhibited a typical hES cell phenotype with a high nucleus to cytoplasmic ratio and prominent nucleoli (Supplemental Figs. 1a–d). Throughout culture all hES cell lines remained OCT4$^+$, Nanog$^+$, TRA1-60$^+$, TRA1-81$^+$, SSEA3$^+$, and SSEA4$^+$ as demonstrated by immunostaining. FACS analysis showed that after 10 passages 97.9% HUES7 and 93.6% HUES1 cells were Nanog$^+$ (Figs. 3a–d) and 93.0% HUES7 and 86.4% HUES1 cells were OCT4$^+$ (Figs. 3e–h). Furthermore, the HUES7 cells exhibited no expression of CD30, a biomarker of transformed human pluripotent stem cells (Herszfeld et al., 2006) (Supplemental Fig. 2). Moreover, karyotypic analysis of hES cells cultured for 10 passages under these conditions exhibited a normal, diploid karyotype (Supplemental Fig. 3) with the appearance of only few sporadic abnormalities (a deletion of 16q and a deletion of 18q have been detected).

hES cell differentiation after long-term culture under modified conditions

To demonstrate the maintenance of differentiative capacity, embryoid bodies were formed by suspension culture of HUES7, MAN1, and HUES1 after culture for >10 passages in the serum/feeder-free system. By Day 1, cell suspensions had formed small cell clusters, which became more regular and compact by Day 2 (Supplemental Fig. 4). By Day 4 the embryoid bodies appeared to have formed a distinct

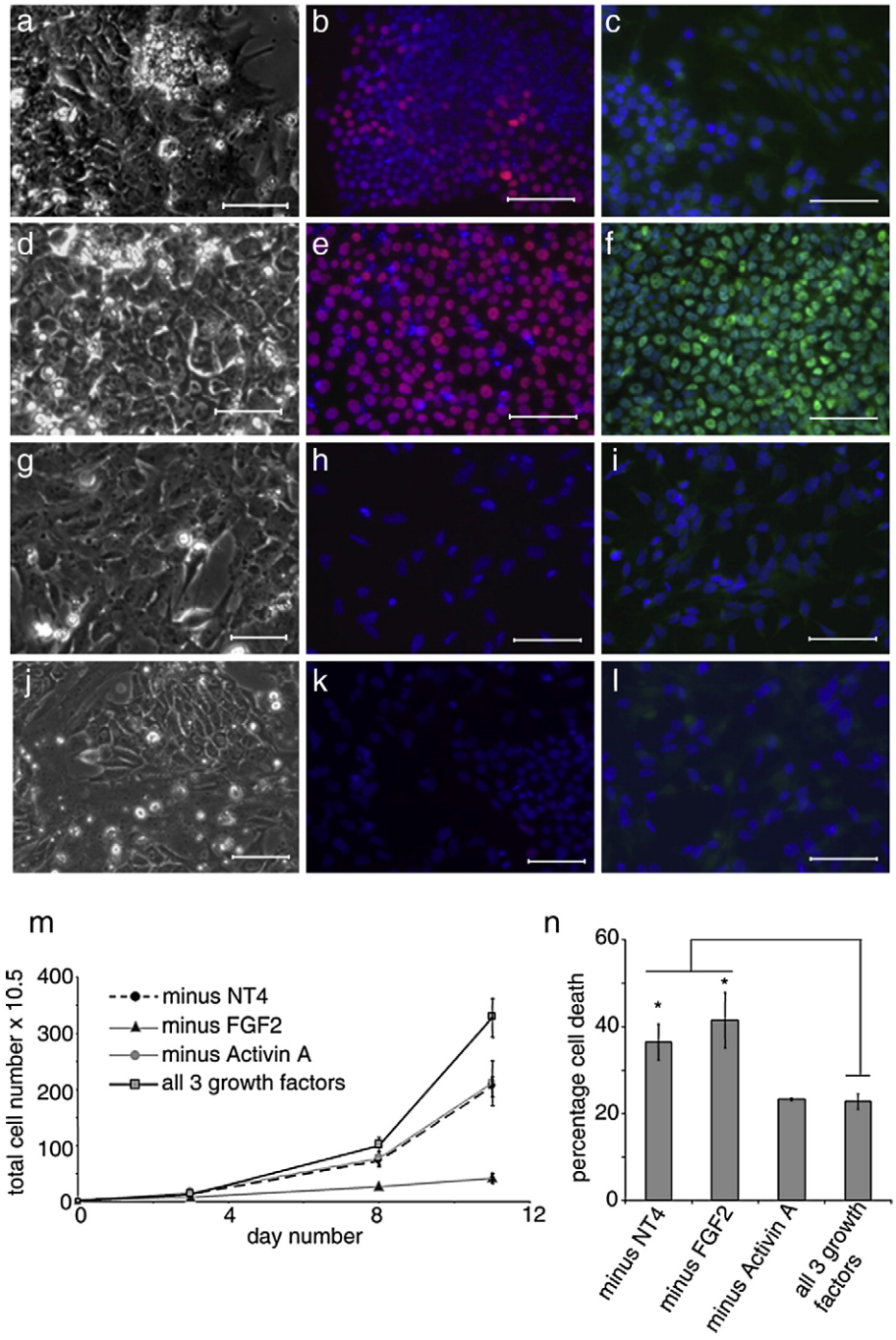

Figure 2 HUES7 cells cultured in the absence of either NT4 (a–c), under the complete feeder/serum-free conditions (d–f), in the absence of activin A (g–i), or in the absence of FGF2 (j–l) for 3 passages for over 2 weeks. Cells were then immunostained for OCT4 (b, e, h, k) and Nanog (c, f, i, l). Scale bars 50 μm. (m) HUES7 culture kinetics determined over 11 days under complete feeder/serum-free conditions with either all growth factors or in the absence of each growth factor ($n=3$). (l) The percentage of cell death determined at 11 days (3 passages), a significant difference in comparison to the control is indicated ($n=3$, $P<0.05$).

peripheral layer reminiscent of the endodermal cell layer of mouse embryoid bodies (Murray and Edgar, 2004) when observed by phase contrast microscopy. By Day 13, the peripheral layer appeared thicker and the inner core more compact. Day 13 embryoid bodies derived from HUES7 cells were cryosectioned and immunostained. The embryoid bodies were $OCT4^-$ and exhibited peripheral AFP^+ cells surrounding a laminin-positive ring of apparent basement membrane (Supplemental Figs. 4g–i). To confirm hES cell pluripotency, Day 13 embryoid bodies (EBs) were plated onto serum-coated glass coverslips and cultured for 2 weeks in serum-containing medium. The differentiating cells from all hES cell lines demonstrated positive immunostaining for ectoderm markers including neurofilament, βIII-tubulin, and GFAP; mesoderm markers brachyury and vimentin and endoderm markers including neurogenin 3, FOXA2, and AFP

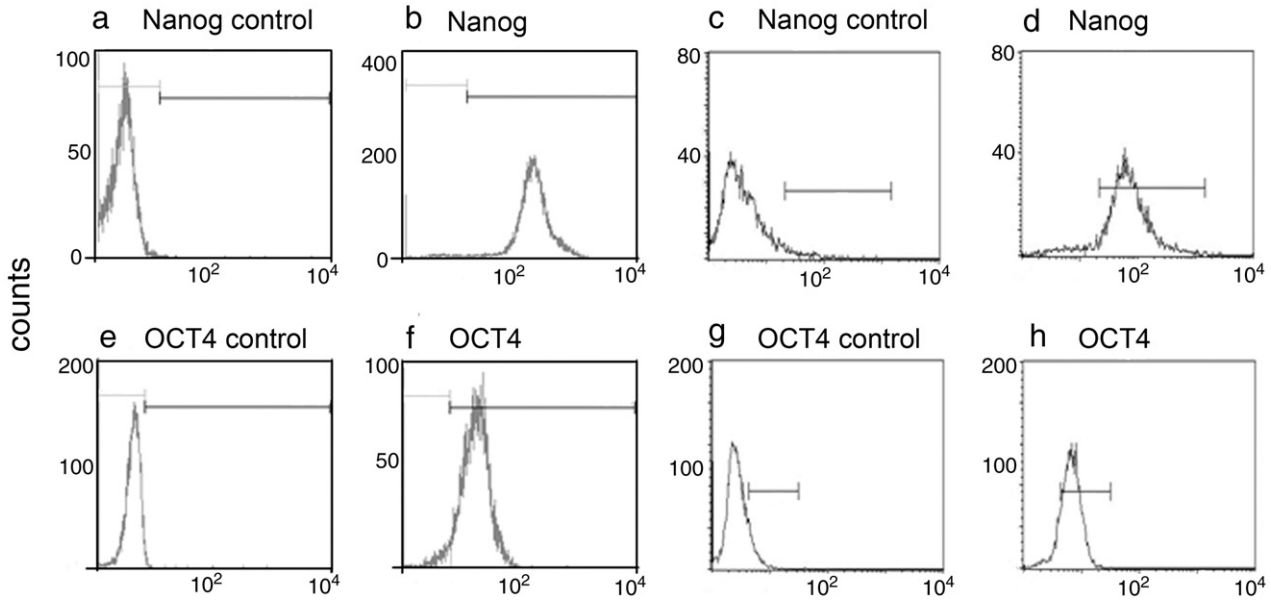

Figure 3 FACS analysis of HUES7 (a, b, e, f) and HUES1 (c, d, g, h) cells after culture for 10 passages under defined feeder-free conditions; Nanog secondary only control (a, c) and Nanog (b, d), OCT4 secondary only control (e, g) and OCT4 (f, h).

(see Fig. 4 and Supplemental Fig. 5). This shows that HUES7, HUES1, and MAN1 hES cell lines remain pluripotent following long-term culture in this feeder/serum-free system.

RT-PCR using human-specific primers was carried out on cDNA from HUES7 cultured with and without feeders for 10 passages, and Day 7 embryoid bodies generated from passage 10, feeder-free HUES7 cultures (Fig. 4). OCT4 was expressed in both types of hES cell culture and by embryoid bodies. By Day 7, embryoid bodies generated from feeder-free HUES7 cultures expressed mesoderm markers *FLK1*, *brachyury*, and

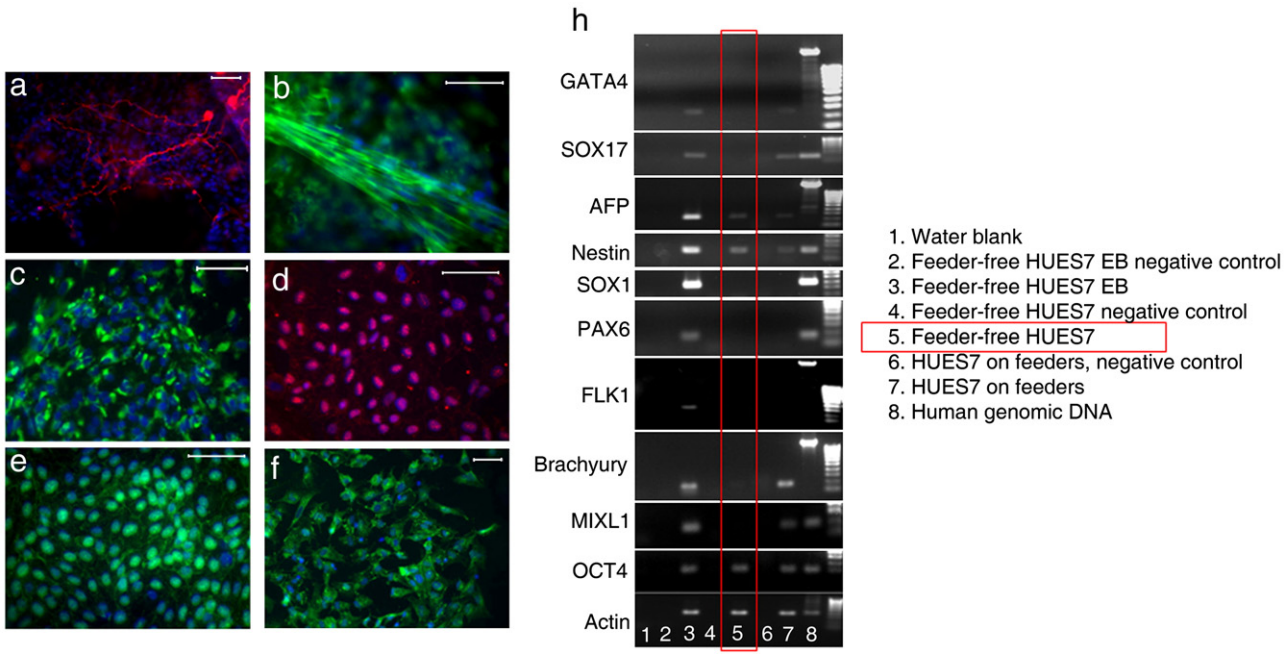

Figure 4 Day 7 embryoid body outgrowths generated from HUES7 cultured for >10 passages under the feeder/serum-free conditions. Outgrowths were immunostained for neurofilaments (a), vimentin (b), glial fibrillary-associated protein (c), neurogenin 3 (d), brachyury (e), and AFP (f). Scale bars 50 μm. Expression of differentiation markers (h). Water blank (1); embryoid bodies derived from HUES7 cultured>10 passages under feeder/serum-free conditions: negative control (2) and cDNA (3); HUES7 cultured>10 passages under feeder/serum-free conditions: negative control (4) and cDNA (5); HUES7 cultured on feeders: negative control (6) and cDNA (7) and human genomic DNA (8).

MIXL1; endoderm markers *AFP*, *GATA4*,and *SOX17*; and ectoderm markers *nestin*, *SOX1*, and *PAX6*. Expression of all these markers other than *nestin* and *AFP* was absent in the undifferentiated feeder-free HUES7 cultures. However, HUES7 harvested after culture on feeders expressed detectable *SOX17*, *GATA4*, *MIXL1*, and *brachyury* as well as *AFP* and *nestin*. As all the primers were human specific (Supplemental Fig. 6); this suggested that a higher level of background differentiation can occur when HUES7 are grown on feeders 5 days after seeding in comparison to the feeder-free system, in which >95% of HUES7 are OCT4 and Nanog positive (Fig. 3). Taken together, these observations demonstrate that hES cells remain pluripotent but retain differentiative capacity following long-term culture in this feeder-free system.

Role of FGF2 in defined culture medium

It has been previously reported that FGF2 supports hES cell culture indirectly by stimulating the production of other supportive factors such as IGFII and TGF-β family members

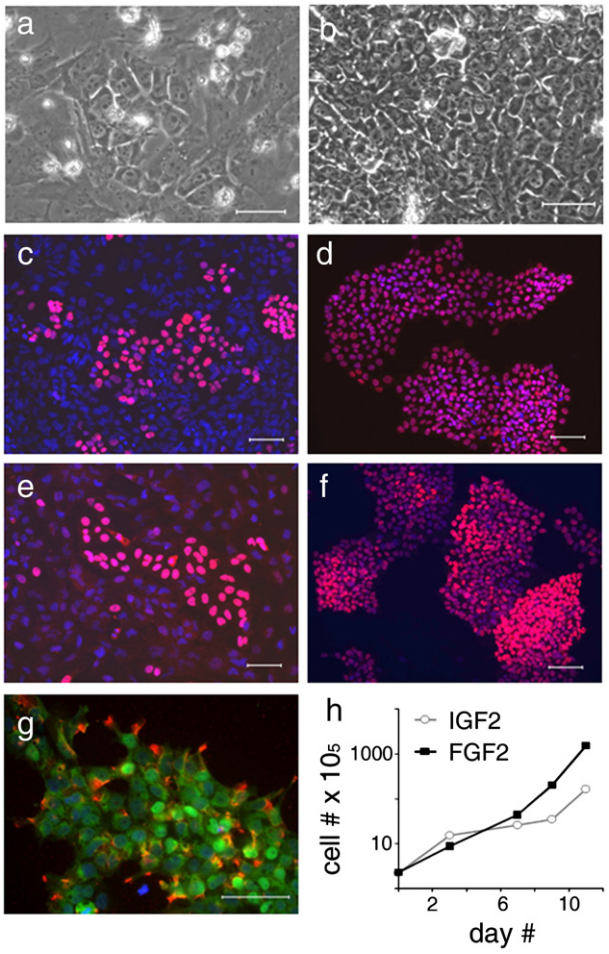

Figure 5 HUES7 cells cultured with IGFII (a, c, e) or FGF2 (b, d, f, g) under the feeder/serum-free conditions: immunostained for OCT4 (c, d) and Nanog (e, f) or costained for both Nanog (green) and FGFR1 (red) (g). Growth kinetics of cultures over 11 days (up to 4 passages) in the presence of either IGFII or FGF2 (h). Scale bars 50 μm.

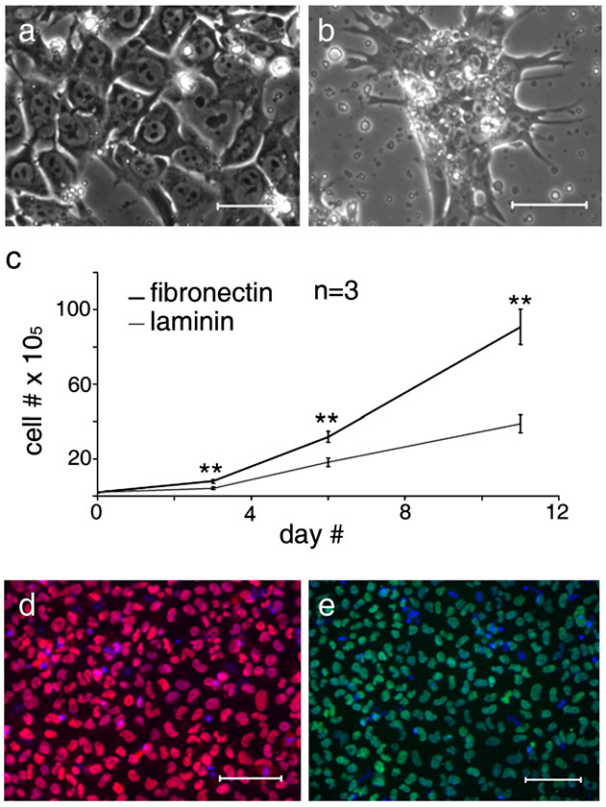

Figure 6 HUES7 cells cultured on either laminin (b, d, e) or fibronectin (a) in the serum-free medium: growth kinetics over 3 passages (c), a significant difference between the two data sets is indicated ($n=3$, $P<0.05$). HUES7 after culture on laminin for 3 passages immunostained for Nanog (d) and OCT4 (e). Scale bars 50 μm.

from fibroblast-like cells generated by hES cells in culture (Bendall et al., 2007). In light of this, we sought to determine whether IGFII could support hES cell culture in the absence of exogenous FGF2 under our more defined feeder/serum-free culture conditions. HUES7 cells were grown for 4 passages over 11 days with the addition of either IGFII or FGF2. In our system, after 4 passages with IGFII cultures appeared morphologically heterogeneous, with small OCT4$^+$, Nanog$^+$ colonies surrounded by OCT4$^-$, Nanog$^-$ fibroblastic-like cells (Fig. 5). In contrast, in the presence of FGF2 cultures exhibited homogeneous hES cell morphology and the majority of cells were OCT4$^+$, Nanog$^+$. Culture growth rate was also slower in the presence of IGFII compared to FGF2 (Fig. 5h). It has also been reported that pluripotent hES cells do not express the FGFR1 receptor; and therefore FGF2 does not act directly on hES cells (Bendall et al., 2007). However, immunostaining of HUES7 under our culture conditions clearly showed a colocalisation of FGFR1 on many Nanog$^+$ cells (Fig. 5g). Together, our results show that in the absence of exogenous FGF2 the addition of IGFII allows the development of a subpopulation of differentiating cells. With the addition of FGF2 in the absence of exogenous IGFII this differentiating subpopulation is absent. Therefore for the maintenance of hES cell cultures the addition of IGFII cannot substitute for the addition of FGF2 under these feeder/serum-free culture conditions.

Role of fibronectin substrates with defined culture medium

To investigate the requirement of hES cell self-renewal on fibronectin substrates, HUES7 cells were cultured for 3 passages on either human placenta laminin or fibronectin in the defined medium. We found no significant difference between the plating efficiency on laminin compared to that on fibronectin (96 ± 1% versus 98 ± 1%, respectively, $P > 0.05$). On fibronectin, hES cells exhibited typical hES cell morphology, a high ratio of nucleus to cytoplasm and prominent nucleoli (Fig. 6a). In contrast, on laminin hES cell colonies were more compact and peripheral cells exhibited multiple processes (Fig. 6b) that were absent on fibronectin. After 3 passages on laminin, the majority of cells still remained Nanog$^+$, OCT4$^+$ (Figs. 6d, e), but the growth rate was significantly slower on laminin compared to that on fibronectin (Fig. 6c). These data show that while both fibronectin and laminin can support hES cell self-renewal, hES cells exhibit a higher proliferative rate on fibronectin.

To investigate the mechanism of interaction between hES cells and the fibronectin substrate, cells cultured under feeder-free conditions on fibronectin were immunostained with antibodies for α5, αV, and β1 integrin. OCT4$^+$ hES cells cultured for >5 passages on fibronectin expressed α5 and β1 integrin receptors (Figs. 7a, b). In addition, Nanog$^+$ hES cells also exhibited active β1 integrin, as determined by costaining with antibody specific to the active form of β1 (Fig. 7c). Immunostaining indicated that Nanog$^+$ hES cells also expressed αV integrin, although the antibody signal was low (Fig. 7d).

The addition of anti-β1 antibody inhibited cell attachment to fibronectin in a dose-dependent manner. The amount of 10 μg/ml anti-β1 blocked all cell attachment whereas after 4 h 85 ± 1% ($n = 3$) of hES cells had attached in the presence of 10 μg/ml isotype control antibody (Fig. 7e). These data suggest that functional β1 integrin is necessary

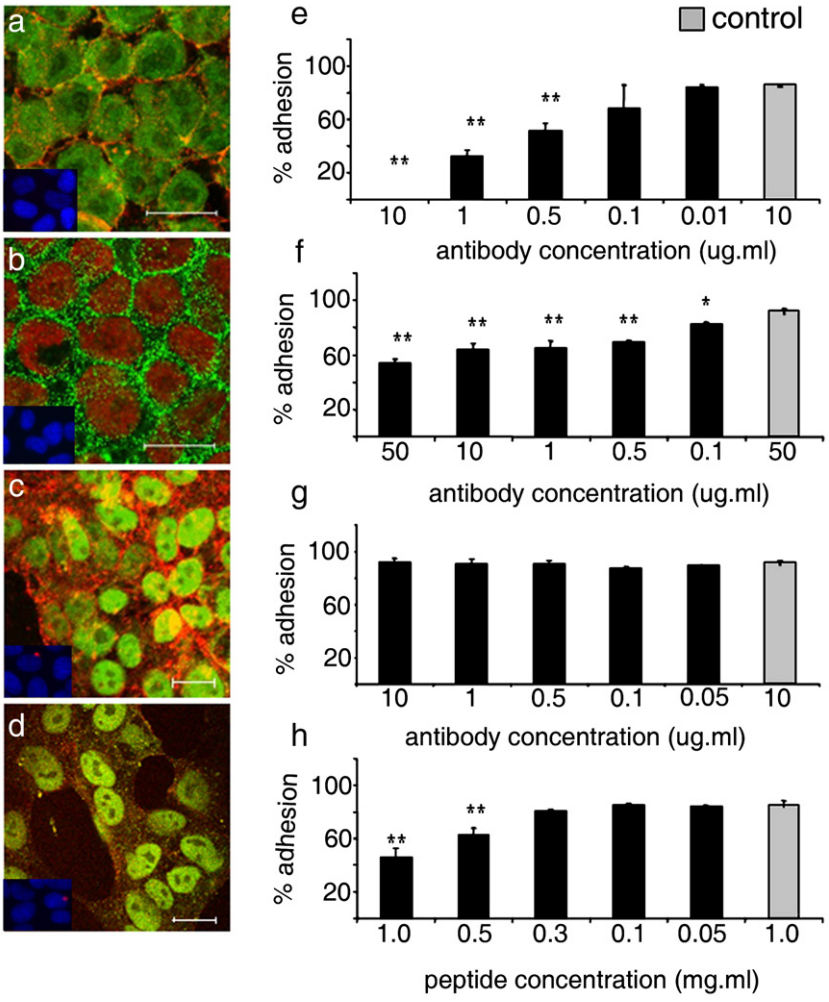

Figure 7 HUES7 coimmunostained for α5 integrin (red), OCT4 (green), inset, α5 integrin isotype control (red) with DAPI counterstaining (a); β1 integrin (green), OCT4 (red), inset, β1 integrin isotype control (green) with DAPI counterstaining (b); active β1 integrin (red), Nanog (green), inset, active β1 integrin (red) with DAPI counterstaining (c) and αV integrin (red), Nanog (green), inset, αV integrin isotype control (red) with DAPI counterstaining (d). Scale bars 15 μm. HUES7 adhesion to fibronectin with anti-β1 integrin antibody (e), α5 integrin antibody (f), αV integrin antibody (g), and RGD peptide (h), a significant difference to the control is shown ($n = 3$, $P < 0.05$).

for the attachment of hES cells to fibronectin. Interestingly, after 24 h HUES7 cultures treated with 10 μg/ml anti-β1 antibody still showed no attachment to the fibronectin; instead cells had clustered into balls resembling early EBs (Supplemental Fig. 7). Addition of anti-α5 integrin reduced cell adhesion in a dose-dependent manner, although only by 46±3% ($n=3$, $P<0.001$) at 50 μg/ml (Fig. 7f). In contrast the addition of anti-αV blocking antibody did not inhibit hES cell adhesion to fibronectin (Fig. 7g). To determine whether the α5 or β1 integrin receptors were interacting with the fibronectin substrate via the RGD tripeptide motif, soluble peptide encoding RGD was added to the culture system. This blocked hES cell adhesion in a dose-dependent manner: 1 mg/ml blocked 54±7% cell adhesion in comparison to only 14±2% in the presence of 1 mg/ml control peptide ($n=3$, $P=0.006$, Fig. 7h).

Discussion

We have used a refined hES cell culture system to examine the individual roles of both growth factors and extracellular matrix molecules in self-renewal and maintenance of pluripotency. Previously published defined, serum/feeder-free culture conditions for the long-term maintenance of pluripotent hES cells have proved difficult to reproduce (Rajala et al., 2007). This may at least be partly explained by the fact that they have often been validated using particular hES cell lines and so may not necessarily be appropriate for all. Indeed we found that two previously published methods (Vallier et al., 2005; Liu et al., 2006) were insufficient to support the commonly used HUES7 hES cell line. However, based on both of these methods we delineate a feeder-free culture system that can support the self-renewal of at least 3 different hES cell lines; HUES7, HUES1, and MAN1.

Under previously reported feeder-free conditions there is evidence that hES cells adapt by differentiating to produce a subpopulation of autologous feeders which may be able to provide the supportive factors necessary to maintain hES cell self-renewal (Bendall et al., 2007). Alternatively a subpopulation of hES cells may themselves adapt to survive under suboptimal culture conditions (Baker et al., 2007). However, the plating efficiency of the hES cell lines when transferred to the present conditions was high (>80%), indicating that there is no apparent selection of a hES cell subpopulation. Furthermore, we did not detect the presence of a feeder cell subpopulation as flow cytometry showed that >95% of cells were OCT4 and Nanog positive. Morphologically, cultures were homogeneous with cells presenting a typical hES cell appearance. RT-PCR also demonstrated the lack of various differentiation marker expressions that were often present when hES cells were cultured on MEF layers. These data suggest that this system can reduce the background differentiation sometimes present in conventional MEF cell cocultures, possibly due to heterogeneity in the microenvironment provided by the MEF cells. It also indicates that our feeder/serum-free culture system provides all the growth factors necessary to maintain long-term hES cell self-renewal.

Our results clearly show that all three growth factor supplements are necessary for long-term hES cell maintenance, and has allowed a better understanding of each of their roles in pluripotency or self-renewal. It has previously been reported that activin A can inhibit hES cell differentiation by activation of SMAD 2/3 signaling (James et al., 2005; Peerani et al., 2007; Pera et al., 2004; Xu et al., 2002). Indeed, we found that in the absence of activin A, hES cells lost pluripotency by 3 passages as shown by a loss of OCT4 and Nanog expression, in addition to a reduction in the rate of hES cell proliferation. Similarly, it has been previously shown that NT4 promotes hES cell survival on MEFs or Matrigel (Pyle et al., 2006) and we demonstrate here that the absence of NT4 reduced cell survival over 3 passages. In the absence of NT4 hES cells lost Nanog and most OCT4 expression, indicating either the selection of differentiated cells due to reduced hES cell survival or an additional role of NT4 in the maintenance of hES cell pluripotency.

Most media for hES cell culture, both on and off feeder cells, are supplemented with FGF2 (Amit et al., 2004). FGF2 may maintain pluripotency by activation of the MEK/ERK and PI3K/AKT signaling cascades (Li et al., 2007) and inhibit differentiation by the suppression of SMAD1 signaling (Peerani et al., 2007). Consistent with this, under our feeder/serum-free culture conditions the withdrawal of FGF2 led to a loss of both Nanog and OCT4 expression. In addition there was decreased cell survival after 3 passages, preceded by a significant reduction in culture growth rate that became noticeable as early as 3 days after FGF2 withdrawal.

It has recently been suggested that FGF2 supports hES cell culture indirectly by stimulating hES cell-derived fibroblast-like cells to produce hES cell-supportive factors such as IGFII and TGFβ family members (Bendall et al., 2007). However we found that in the absence of exogenous FGF2, the addition of IGFII facilitated hES cell differentiation, leading to the generation of an $OCT4^-$, $Nanog^-$ subpopulation of cells that surrounded hES cell colonies. In contrast, in the presence of FGF2 this subpopulation of fibroblast-like cells was not observed. Furthermore the presence of FGFR1 on $Nanog^+$ hES cells (Fig. 5g) suggested that FGF2 can act directly on these cells. This has also been reported by Schuldiner et al. (2000). Our data therefore suggest that FGF2 does not maintain hES cell self-renewal via the stimulation of factors from a subpopulation of fibroblast-like cells under our conditions. The discrepancies between our observations and those of Bendall et al. are likely to result from different culture conditions: the substrate used in our study was purified human plasma fibronectin, whereas previously mouse Matrigel was used (Bendall et al., 2007). Matrigel contains a variety of different biologically active factors (Kleinman et al., 1982), any of which may induce background differentiation that might explain the observation of a differentiated subpopulation (Bendall et al., 2007). This highlights the importance of developing more defined culture conditions to allow clearer interpretation of hES cell responses.

Our results demonstrate the requirement for appropriate human extracellular matrix substrate for hES cell self-renewal in the absence of serum and feeder cells. As laminin has been reported to support pluripotent hES cell culture we compared human plasma fibronectin with human placenta laminin. We found that while fibronectin and laminin could both support self-renewal, hES cell culture growth was faster on fibronectin. These data suggest that human plasma fibronectin may be a better substrate for hES cell

maintenance, in terms of proliferation rates, lack of spontaneous differentiation, and scalability in comparison to human placenta laminin. The fact that both could support hES cell culture to some extent indicates some redundancy in the ability of ECM protein substrates to support hES cell self-renewal. Indeed vitronectin has also been shown to maintain hES cell self-renewal (Braam et al., 2008), albeit with some spontaneous differentiation necessitating mechanical dissociation to allow the removal of differentiated cells. Although we have not examined whether vitronectin could support hES cell culture with our medium, Lui et al. have reported that with their medium (which includes 100 ng/ml FGF2, the N2 and B27 supplements but no activin A or NT4) vitronectin could not support hES cell for 7 days (Liu et al., 2006).

The mechanism by which hES cells interact with and are maintained by such substrates has been little investigated and here we start to decipher some of the key cellular receptors. The $\alpha5\beta1$ integrin is a major receptor for fibronectin (Hayashi et al., 2007) and under the feeder/serum-free conditions hES cells expressed both the $\alpha5$ and the active $\beta1$ integrin receptor subunits, as previously shown when cultured on feeder cells (Braam et al., 2008). Importantly, a functional role of the integrin could be demonstrated by inhibition of hES cell attachment to fibronectin substrates with an RGD peptide and with blocking antibodies to either of the integrin subunits. In contrast to total inhibition with $\beta1$ antibodies, there was only a partial inhibition with anti-$\alpha5$, consistent with the existence of alternative integrin α subunits that can bind fibronectin. Indeed the αV subunit normally present on mES cells has been shown to functionally compensate for the loss of the $\alpha5$ subunit in $\alpha5$ null mES cells plated on fibronectin (Yang and Hynes, 1996). However, we found no inhibition of cell attachment to fibronectin with anti-αV blocking antibody. The addition of soluble RGD peptide blocked some but not all adhesion, suggesting that alternative fibronectin binding sites might also mediate hES cell adhesion to fibronectin (Mostafavi-Pour et al., 2001). These data are consistent with the recent observation that the $\alpha V\beta5$ integrin functions as a receptor for vitronectin substrates (Braam et al., 2008). However, the αV subunit does not appear to be involved with hES cell/fibronectin interactions, and so other α subunits (for example, $\alpha4$) may be involved. Interestingly, cell to cell adhesion appeared unaffected by blocking anti-$\beta1$ integrin antibody (since the nonattached cells were able to aggregate). Similarly previous reports showed that $\beta1$ integrin-deficient mES cells also do not exhibit impaired cell to cell adhesion and are able to integrate normally into the inner cell mass (Fassler et al., 1995). It is well recognised that cell behavior is regulated by the cross-talk between signaling pathways dependent on integrin-mediated cell adhesion and growth factors (Schwartz and Ginsberg, 2002). Future work will analyse such cross-talk in the hES cells leading to their self-renewal.

The feeder/serum-free culture conditions described here already exploit this cross-talk to provide a defined system for investigation into hES cell-ECM interactions, which result in the elimination of essentially all hES cell spontaneous differentiation. This work therefore represents an important advance in understanding the role of soluble growth factors and substrate-bound components in hES cell maintenance.

Materials and methods

Human ES cells

Cleavage stage embryos were donated after informed consent by couples undergoing IVF treatments at St. Mary's Hospital. Embryos were cultured to blastocyst stage in G3 series sequential media (Vitrolife, Edinburgh, UK). At blastocyst stage, the zona pellucida was removed by treatment with acid Tyrode's solution (Sigma), and denuded blastocysts were washed in G2 media (Vitrolife). The trophectoderm (TE) was partially removed mechanically and the inner cell mass seeded onto MEFs. This was cultured for 8 days and then the outgrowth was passaged by manual dissection for the initial 10 passages (MAN1). HUES1 and 7 were obtained from Harvard University (hES Cell Facility/Melton Laboratory, MA, USA).

hES cell culture on feeder cells

Murine embryonic fibroblast feeder cells (MEFs) were prepared from Day 13.5 CD1 mouse embryos, cultured in F-DMEM (DMEM (Lonza, Nottingham, UK) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 1× penicillin/streptomycin (Invitrogen, Paisley, UK), and mitotically inactivated at passage 3–5 with 10 μg/ml mitomycin C (Sigma, Dorset, UK). The hES cells lines HUES7, HUES1, and MAN1 were cultured on MEFs plated at a density of 6×10^4 cm^{-2} on 0.1% gelatin (Sigma)-coated dishes 24 h previously. The hES cells were cultured on MEFs in knockout Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% (v/v) knockout serum replacement (KSR), 2 mM L-glutamine, 1% (v/v) nonessential amino acids (NEAA), 0.1 mM β-mercaptoethanol, 1% (v/v) penicillin/streptomycin (all Invitrogen) and 10 ng/ml FGF2 (Autogen Bioclear, Wiltshire, UK). They were passaged using 0.25% (w/v) trypsin/1 mM EDTA (Invitrogen) as previously described (Ellerstrom et al., 2007).

Feeder-free hES cell culture

The hES cells were initially cultured in either the serum-free culture medium previously described by Lui et al. (DMEM/F12, N-2 and B-27 supplements, 1 mM L-glutamine, 1% NEAA, 0.1 mM β-mercaptoethanol, and 100 ng/ml bFGF (Invitrogen) (Liu et al., 2006) or the serum-free medium previously described by Vallier et al. (50:50 IMDM:F12 NUT MIX (Invitrogen), 7 μg/ml insulin, 15 μg/ml transferrin (Roche, Welwyn Garden City, UK), 0.45 mM monothioglycerol, 5 mg/ml bovine serum albumin factor V (Sigma), 10 ng/ml activin (Preprotech, London, UK), and 12 ng/ml FGF2 (Vallier et al., 2005). Subsequently, for our feeder/serum-free culture conditions, hES cells were cultured in 50:50 F12:DMEM (Lonza) supplemented with 2 mM L-glutamine, 1% NEAA, 0.1 mM β-mercaptoethanol, 1% penicillin/streptomycin, 0.1% bovine serum albumin (BSA) (Sigma), N2 supplement (consisting of 10 g/L transferrin, 500 mg/L insulin, 0.63 mg/L progesterone, 1.611 g/L putrescine), B27 supplement (components described by Brewer et al. (Brewer et al., 1993); concentration of components are not publicly disclosed) (both from Invitrogen), 10 ng/ml activin A, 4 ng/ml neurotrophin 4

(Preprotech, London, UK), and 40 ng/ml FGF2 on 3.5-, 6-, or 10-cm plates coated overnight with a 50 μg/ml human plasma fibronectin (Millipore, Hertfordshire, UK) solution at 4 °C. For the initial 2 passages under the feeder/serum-free conditions cells were more resistant to dissociation, perhaps because of carryover of feeder cells, so trypsin was used the dissociate cultures. Once hES cell cultures were established under the feeder/serum-free conditions, i.e., they had undergone over 3 passages, cell dissociation solution (CDS) was used instead to avoid the problem of variability between different batches of trypsin. Plating efficiency was significantly lower after cells were dissociated with CDS compared to trypsin (71 ± 5% versus 98 ± 1%, respectively, $P<0.02$). Following dissociation, cells were reseeded at a density of 6×10^4 cm^{-2}. Cell number was determined using a hemocytometer and the percentage of dead cells determined using the trypan blue (Sigma) exclusion assay. To assess plating efficiency hES cells on feeders were dissociated with trypsin or CDS and counted before seeding under feeder/serum-free conditions. After 5 h the numbers of unattached cells in the medium were counted and subsequently the percentage of attached cells was calculated.

In vitro hES cell differentiation

For embryoid body formation hES cells were dissociated using trypsin/EDTA and cultured for 7 or 13 days in F-DMEM in bacteriological-grade culture dishes. Day 13 EBs were fixed for 15 min in 4% paraformaldehye (Sigma). They were then embedded in gelatin (7.5% gelatin in a 15% sucrose (Sigma) solution), embedded in O.C.T. embedding compound, frozen in liquid nitrogen, and sectioned. For extended 2D in vitro differentiation, Day 7 EBs were plated on glass coverslips coated with 0.1% gelatin in F-DMEM for a further 14 days.

Immunocytochemistry

For immunostaining cells were fixed in a 4% paraformaldehyde solution for 10 min at room temperature. Cultures were then blocked for 20 min in PBS with 10% serum (of the animal in which the secondary antibody was raised) in PBS. Cells were incubated overnight at 4 °C with primary or relevant isotype control antibodies (Autogen Bioclear, Wiltshire, UK) in 1% serum in PBS. Primary (and relevant isotype control) antibody working concentrations were OCT4 2.5 μg/ml, paxillin 1:200 (BD), Nanog 2 μg/ml, SSEA4 10 μg/ml, SSEA3 10 μg/ml, glial fibrillary acidic protein (GFAP) 5 μg/ml, brachyury 20 μg/ml, vimentin 5 μg/ml, FOXA2 10 μg/ml, neurogenin 3 10 μg/ml, FGFR1 1:100 (R&D Systems, Abingdon, UK), TRA-1-60 10 μg/ml, TRA-1-81 10 μg/ml (Abcam, Cambridge, UK), pan-specific laminin 1:100, vinculin 1:400 (Sigma), α-fetoprotein (AFP) 1:400 (ICN Biomedicals Inc, Basingstoke, UK), neurofilament 1:500 (Biomol International, Exeter, UK), αV 10 μg/ml (Calbiochem). MAB11 and MAB13 (anti-α5 and β1 integrin, respectively) were the kind gift of Ken Yamada, (NIH). Anti-activated β1 integrin 12G10 was a kind gift from Prof. M Humphries (Wellcome Matrix Centre, University of Manchester). For intracellular antigens, 0.1% Triton X-100 (Sigma) was added throughout the staining procedure. Cells were then incubated for 2 h with 4 μg/ml relevant secondary Alexafluor^{488} or Alexafluor^{594}-conjugated antibodies (Invitrogen). Cells were mounted in Vectashield hardset mounting medium with DAPI (Vector, Peterborough, UK).

Flow cytometry

Single hES cell suspensions were fixed in ice-cold methanol for 10 min and then permeabilised in a 0.1% Triton X-100 solution for 15 min. Samples were stained with 50 μg/ml anti-OCT4 or anti-Nanog overnight at 4 °C followed by 1 h with the appropriate Alexafluor^{488}-conjugated secondary antibody (4 μg/ml). For controls, cell samples were stained with secondary antibody only. Flow cytometric analysis was performed on a Beckman Coulter Cell Lab Quanta SC machine and at least 20,000 events were collected. The data were analysed using BC Cell Lab Quanta SC MPL analysis software.

Reverse-transcriptase PCR

RNA was isolated using TRI reagent (Sigma) according to the manufacturer's protocol and subsequently treated with Dnase I (Sigma). RNA was reverse-transcribed using SuperScript II reverse transcriptase and random primers (Invitrogen). PCR was carried out using Biotaq DNA polymerase (Bioline, London, UK) according to the manufacturer's protocol. Briefly, samples were denatured for 5 min at 94 °C and then cycled 30 times at 94 °C for 30 s, 50–65 °C for 30 s, and 72 °C for 30 s before a final extension at 72 °C for 10 min. Primers were designed to amplify *OCT4*, and markers for endoderm (*AFP, SOX17*, and *GATA4*) mesoderm (*brachyury, MIXL1, FLK*1) and ectoderm (*SOX1, nestin, PAX6*). β-*Actin* and murine *GAPDH* were amplified as controls. Primer sequences, annealing temperature, expected size, and gene accession number are listed in Supplemental Table 1. RT PCR for the canonical form of CD30 expression was carried out as published by Herszfeld et al. (2006) using NTERA2 (kind gift from Professor P. Andrews, University of Sheffield) cDNA as a positive control.

Karyotyping

hES cells cultured under our feeder/serum-free conditions for >10 passages were treated with 10 μg/ml colchicine (Sigma) for 2 h at 37 °C. Cells were then dissociated with trypsin and treated with hypotonic solution. (27.5% PBS^+ solution) for 15 min at 37 °C. Samples were then fixed with Carnoy's fixative. Cell samples were then G-banded and 24 metaphase spreads were examined for karyotypic abnormalities by TDL, London, UK, and the North West Regional Cytogenetics Unit.

Adhesion assays

Cells were preincubated with MAB11, MAB13, anti-αV or appropriate isotype control in culture medium for 30 min at 37 °C. For adhesion assays using GRGDSP peptide and the GRGQSP control peptide (Bachem, Merseyside, UK) cells were preincubated with peptide in culture medium for 15 min at 37 °C. Cells were then seeded at a density of 5×10^4 cm^{-2} on fibronectin-coated plastic and incubated for a

further 4 h for antibody assays, or 2.5 h for the peptide assay, at 37 °C. The percentage adhesion was calculated by counting the number of unattached cells. Statistical significance was assessed using the Student unpaired *t* test.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.scr.2009.03.002.

References

Amit, M., et al., 2000. Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. Dev. Biol. 227, 271–278.

Amit, M., et al., 2004. Feeder layer- and serum-free culture of human embryonic stem cells. Biol Reprod 70 (3) 837–845.

Baker, D.E.C., et al., 2007. Adaptation to culture of human embryonic stem cells and oncogenesis in vivo. Nat. Biotechnol. 25 (2), 207–215.

Bendall, S.C., et al., 2007. IGF and FGF cooperatively establish the regulatory stem cell niche of pluripotent human cells in vitro. Nature 448 (7157), 1015–1021.

Braam, S.R., et al., 2008. Recombinant vitronectin is a functionally defined substrate that supports human embryonic stem cell self renewal via Avb5 integrin. Stem Cells 26 (9), 2257–2265.

Brewer, G.J., et al., 1993. Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. J. Neurosci. Res. 35 (5), 567–576.

Chin, A.C., et al., 2007. Identification of proteins from feeder conditioned medium that support human embryonic stem cells. J. Biotechnol. 130 (3), 320–328.

Ellerstrom, C., et al., 2007. Facilitated expansion of human embryonic stem cells by single-cell enzymatic dissociation. Stem Cells 25 (7), 1690–1696.

Fassler, R., et al., 1995. Lack of beta 1 integrin gene in embryonic stem cells affects morphology, adhesion, and migration but not integration into the inner cell mass of blastocysts. J. Cell. Biol. 128 (5), 979–988.

Hayashi, Y., et al., 2007. Integrins regulate mouse embryonic stem cell self-renewal. Stem Cells 25 (12), 3005–3015.

Herszfeld, D., et al., 2006. CD30 is a survival factor and a biomarker for transformed human pluripotent stem cells. Nat. Biotechnol. 24 (3), 351–357.

Hisamatsu-Sakamoto, M., Sakamoto, N., Rosenberg, A.S., 2007. Embryonic stem cells cultured in serum free medium acquire bovine apolipoprotein B-100 from feeder cell layers and serum replacement medium. Stem Cells 26 (1), 72–78.

Inzunza, J., et al., 2005. Derivation of human embryonic stem cell lines in serum replacement medium using postnatal human fibroblasts as feeder cells. Stem Cells 23 (4), 544–549.

James, D., et al., 2005. TGFbeta/activin/nodal signaling is necessary for the maintenance of pluripotency in human embryonic stem cells. Development 132 (6), 1273–1282.

Kleinman, H.K., et al., 1982. Isolation and characterization of type IV procollagen, laminin, and heparan sulfate proteoglycan from the EHS sarcoma. Biochemistry 21 (24), 6188–6193.

Li, J., et al., 2007. MEK/ERK signaling contributes to the maintenance of human embryonic stem cell self-renewal. Differentiation 75 (4), 299–307.

Liu, Y., et al., 2006. A novel chemical-defined medium with bFGF and N2B27 supplements supports undifferentiated growth in human embryonic stem cells. Biochem. Biophys. Res. Commun. 346 (1), 131–139.

Ludwig, T.E., et al., 2006. Derivation of human embryonic stem cells in defined conditions. Nat. Biotechnol. 24 (2), 185–187.

Lu, J., et al., 2006. Defined culture conditions of human embryonic stem cells. PNAS 103 (15) 5688–5693.

Martin, M.J., et al., 2005. Human embryonic stem cells express an immunogenic nonhuman sialic acid. Nat. Med. 11 (2), 228–232.

Mostafavi-Pour, Z., et al., 2001. Identification of a novel heparin-binding site in the alternatively spliced IIICS region of fibronectin, roles of integrins and proteoglycans in cell adhesion to fibronectin splice variants. Matrix Biol. 20 (1), 63–73.

Murray, P., Edgar, D., 2004. The topographical regulation of embryonic stem cell differentiation. Philos. Trans. R. Soc. Lond. B Biol. Sci. 359 (1446), 1009–1020.

Peerani, R., et al., 2007. Niche-mediated control of human embryonic stem cell self-renewal and differentiation. EMBO J. 26 (22), 4744–4755.

Pera, M.F., et al., 2004. Regulation of human embryonic stem cell differentiation by BMP-2 and its antagonist noggin. J. Cell Sci. 117 (Pt 7), 1269–1280.

Pyle, A.D., Lock, L.F., Donovan, P.J., 2006. Neurotrophins mediate human embryonic stem cell survival. Nat. Biotechnol. 24 (3), 344–350.

Rajala, K., et al., 2007. Testing of nine different xeno-free culture media for human embryonic stem cell cultures. Hum Reprod 22 (15), 1231–1238.

Richards, M., et al., 2003. Comparative evaluation of various human feeders for prolonged undifferentiated growth of human embryonic stem cells. Stem Cells 21 (5), 546–556.

Richards, M., et al., 2002. Human feeders support prolonged undifferentiated growth of human inner cell masses and embryonic stem cells. Nat. Biotechnol. 20 (9), 933–936.

Schuldiner, M., et al., 2000. Effects of eight growth factors on the differentiation of cells derived from human embryonic stem cells. PNAS 97 (21), 11307–11312.

Schwartz, M.A., Ginsberg, M.H., 2002. Networks and crosstalk: integrin signalling spreads. Nat. Cell Biol. 4 (4), E65–E68.

Stojkovic, P., et al., 2005. An autogeneic feeder cell system that efficiently supports growth of undifferentiated human embryonic stem cells. Stem Cells 23 (3), 306–314.

Thomson, J.A., et al., 1998. Embryonic stem cell lines derived from human blastocysts. Science 232 (5391), 1145–1147.

Vallier, L., Alexander, M., Pedersen, R.A., 2005. Activin/Nodal and FGF pathways cooperate to maintain pluripotency of human embryonic stem cells. J Cell Sci 118 (19), 4495–4509.

Xu, C., et al., 2005. Basic fibroblast growth factor supports undifferentiated human embryonic stem cell growth without conditioned medium. Stem Cells 23 (3), 315–323.

Xu, C., et al., 2001. Feeder-free growth of undifferentiated human embryonic stem cells. Nat. Biotechnol. 19 (10), 971–974.

Xu, R.H., et al., 2002. BMP4 initiates human embryonic stem cell differentiation to trophoblast. Nat. Biotechnol. 20 (12), 1261–1264.

Yang, J.T., Hynes, R.O., 1996. Fibronectin receptor functions in embryonic cells deficient in alpha 5 beta 1 integrin can be replaced by alpha V integrins. Mol. Biol. Cell 7 (11), 1737–1748.

Yao, S., et al., 2006. Long-term self-renewal and directed differentiation of human embryonic stem cells in chemically defined conditions. PNAS 103 (18), 6907–6912.

HUES Cell Collection Instruction Manual. http://mcb.harvard.edu/melton/hues/HUES_manual.pdf.